REMARKS

This is in response to the Office Action dated November 14, 2006 in the above-identified application. Applicants request a one-month extension of time for response and enclose the required fee.

The specification has been objected to because of an informality in paragraph [0008] on page 4. The requested correction has been made in the substitute paragraph [0008] attached. Please note that the author is Dan G. Fraenkel and the journal is *E. coli and Salmonella*, volume 12, pages 142-150, 1996.

Claims 1-5, 8-13, 23 and 24 are currently pending. Claims 6, 7 and 14-22 have been withdrawn as directed to a non-elected invention. By this response, Applicants have amended Claims 1, 9 and 24 and have canceled Claims 8 and 10-12.

Claim 13 has been objected to as being broader than Claim 9 from which it depends. As discussed in detail below, *E. coli* strain FTR2717 (Claim 13) is derived from parental strain TRN212, which comprises the endogenous and exogenous genes of the strain of Claim 9. Thus Claim 13 is not broadening and properly depends from Claim 9 as a particular *E. coli* strain.

Claim 1 has been rejected under 35 USC §101 as allegedly defining a product of nature and, thus, non-statutory subject matter. Claim 1 has been amended to provide that the claimed *E. coli* is isolated and purified – thus, not a product of nature. *See In re Bergy*, 563 F.2d 1031, 195 USPQ 344 (CCPA 1977). In view of the amendments to Claim 1, Applicants maintain that the rejection has been overcome and should be withdrawn.

Claims 9-12 have been rejected under 35USC§112 ¶1 as failing the written description requirement. The Examiner argues that Claim 8 requires an inactivated chromosomal pckA gene and that Claims 9-12, which depend from Claim 8 require the strain to have endogenous and exogenous ppc genes. The Examiner further alleges that the specification teaches deleting the endogenous ppc gene, but not adding the exogenous ppc gene or how to inactivate it. The Examiner further argues that the process of Claim 2 does not produce a ppc gene that codes for a polypeptide having ppc enzyme activity and thus the described strains lack endogenous and exogenous ppc gene(s). The Examiner requests that Applicants point to the specification (page/paragraph) where the description of the claimed is set forth.

Applicants have canceled Claims 8 and 10-12 and amended Claim 9 to be dependent on Claim 1. As provided in the specification, the *E. coli* strain of the present application (FTR2717) was derived from a parent *E. coli* strain TRN212 (Accession No.: KCCM-10353), (see, e.g. paragraph [0020], p. 7, paragraph [0039], p. 15, paragraph [0041], p. 16 and paragraph [0044], p. 17), which in turn, was derived from *E. coli* strain pGmTN-PPC12 (Accession No.: KCCM-10236). *E. coli* strain TRN212, like its parent, *E. coli* strain pGmTN-PPC12, has resistance to threonine, lysine, isoleucine and methionine analogues. The chromosomes of both strains contain an endogenous ppc gene and an endogenous threonine operon containing thrA, thrB and thrC genes, as well as a copy of an exogenous ppc gene and exogenous thrA, thrB and thrC genes. Strain TRN212 has an inactivated tdcBC operon (which is not inactivated in the parent strain pGmTN-PPC12).

A detailed description of *E. coli* strain TRN212 is found in Korean Patent Application No. 2002-015380 disclosed in the present application at paragraph [0020], p. 7, and incorporated by reference into the present specification (see paragraph [0047], p. 19). KR 2002-015380 discloses the methods for preparing the parent strain of the strain of the present invention having the above recited characteristics and also discloses addition to the strain of an exogenous ppc gene coding for a polypeptide having ppc enzyme activity[1].

Certificates of Deposit of the *E. coli* strain FTR2717 of the present invention, as well as parental strains TRN212 and pGmTN-PPC12, are attached, together with a Declaration of Biological Deposit signed by the undersigned.

In view of the amendments and remarks herein, as well as the deposits of the relevant bacterial strains, applicants request that the written description rejection be withdrawn.

Claims 5, 13 and 23 have been rejected under 35 USC § 112, ¶1 as lacking enablement. The Examiner argues that *E. coli* strain TR 2717 is not enabled, because its parent strain TRN212 is not publicly available.

Applicants disagree and provide herein copies of the relevant deposit certificates and a Declaration of Biological Deposit. In view of this submission, Applicants request that the enablement rejection be withdrawn.

[1] Please note that EP 1347057, which is of record is the present case, corresponds to KR 2002-015380 and describes the construction and properties of strain TRN212.

Claims 9-13 have been rejected under 35 USC § 112 ¶2 as indefinite. Claims 10-12 have been canceled. The Examiner alleges that the use of "resistance to" in the claims is unclear.

Applicants disagree. As would be very clear to a person skilled in the art, the term "resistance to" means that the *E. coli* strain of the invention can survive and grow in culture in medium in which the specified amino acid analogues are present. Microbiologists and geneticists understand the meaning of "resistance" as such, for example, antibiotic resistance is where a microorganism can grow in the presence of a specified antibiotic. As a further showing that the meaning is not unclear and the term "resistant to" is not indefinite, the same term appears in claims of other U.S. patents (see, e.g., U.S. Patent 5,217,883 (Abstract, Specification Claim 1) and 5,087,566 (Abstract, Specification, Claim 1) which deal with microorganisms having resistance to amino acids and analogues thereof. Because the term "resistant to" is not unclear, Applicants request that the indefiniteness rejection be withdrawn.

Claims 1-4 and 8 have been rejected under 35 USC § 103(a) as obvious in view of Park et al. (EP 1347057A1, published 9/24/03) and U.S. Patent No. 5,919,694 to Sujimoto issued 7/6/99. Claim 8 has been canceled.

As noted above, EP 1347057 discloses the construction and characteristics of parental strain TRN212 and Sujimoto teaches an *E. coli* having a mutant ppc gene that provides for feedback inhibition by aspartic acid.

The present application claims priority to Korean Patent Application No. 10-2003-0021458, filed April 4, 2003. Priority has been perfected in the present application

(a certified copy of the Korean Priority application has been filed). Applicants submit herewith an English translation of the Korean priority document. As shown in the attached, which fully discloses the present invention, the present invention was made prior to the publication of Park et al., thereby removing Park et al. as a prior art publication. Since Park et al. cannot be prior art to the present application, its combination with Sujimoto is also mooted in rendering Claims 1-4 obvious.

In view of the above remarks, Applicants request that the rejection of Claims 1-4 as obvious be withdrawn.

Applicants believe that no additional fees (other than for a one-month extension of time) are required in connection with this response. However, if additional fees are required, the Commissioner is hereby authorized to charge any additional payment, or credit any overpayment, to Deposit Account No. 01-2300, **referencing Docket Number 027707.00013.**

Respectfully submitted,

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DECLARATION

I, Min Son, of 19th Floor, City Air Tower 159-9 Samseong-dong, Gangnam-gu, Seoul, Korea do hereby solemnly and sincerely declare as follows:

1. That I am well acquainted with the English and Korean languages;
2. That the following is a correct translation into English of the accompanying certified copy of the Korean Patent Application No. 10-2003-0021458.

I execute this solemn declaration consciously believing the above to be true.

Seoul, March 08, 2007

M. Son

Min Son, Ph.D.
Patent Attorney

tdcBC/pckA GENE-INACTIVATED MICROORGANISM AND METHOD OF PRODUCING L-THREONINE USING THE SAME

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to a microorganism that produces L-threonine and a method of producing L-threonine using the microorganism. More particularly, the present invention relates to a microorganism that contains both *tdcBC* and *pckA* genes inactivated on chromosome and is remarkably improved in the productivity of L-threonine due to the inactivation of the two genes, and a method of producing L-threonine using such a microorganism.

Description of the Related Art

L-threonine is known to be an essential amino acid, which has been widely used as an additive to animals' fodder and foods and an animal growth stimulator, as well as a component of medical aqueous solutions and other raw material for medicinal products. L-threonine is currently produced by only five companies in advanced countries, including the Ajinomoto Company in Japan, and is two to three times more expensive than lysine that is known to be highly valuable due to it high price of 5,000-6,000 dollars per ton in the international market. Thus, L-threonine has high growth potential in the world market.

L-threonine is currently produced by only microbial fermentation techniques, using mainly mutants derived from wild types of microorganisms, including *Escherichia coli*, the genus *Corynebacterium*, the genus *Brevibacterium*, the genus *Serratia* and the genus *Providencia*. Examples of these mutants include those having resistance to amino

acid analogues or drugs, and their auxotrophs for diamino-pimelic acid, methionine, lysine and isoleucine (Japanese Pat. Publication No. Heisei 2-219582; Korean Pat. Application No. 1998-32951; *Appl. Microbiol. Biotechnol.*, 29:550-553, 1988). However, such mutant strains are disadvantageous in terms of having low L-threonine productivity and being grown only in media supplemented with expensive diamino-pimelic acid or isoleucine due to their auxotrophic properties for the diamino-pimelic acid or isoleucine. That is, in the case of using a mutant requiring diamino-pimelic acid for growth, this fermentative production of L-threonine requires high costs. Likewise, in case of using an isoleucine auxotroph, a fermentation medium for this auxotroph must be supplemented with expensive isoleucine, resulting in increased production costs of L-threonine.

These problems can be overcome by employing an isoleucine-leaky mutant as disclosed in Korean Pat. Publication No. 92-8365, which does not need isoleucine in a medium and produces high levels of L-threonine than known strains. However, this classical mutation method is also time-consuming and ineffective in selecting novel bacterial strains capable of producing high levels of L-threonine, and has the greatest disadvantage of being limited in improvement of the L-threonine productivity.

In this regard, instead of employing the auxotrophs, other methods for mass production of L-threonine have been developed, which employ recombinant L-threonine-producing microorganisms that have increased activity of enzymes participating in the biosynthesis of L-threonine by metabolic engineering techniques. That is, genes corresponding to enzymes involving in L-threonine metabolism are isolated using genetic recombination techniques, cloned into proper gene vehicles, and introduced into microbial mutants to improve L-threonine productivity of the mutants.

The present inventors previously developed a method of developing a L-threonine producing strain using such metabolic engineering techniques, as disclosed in Korean Pat. Application No. 2001-6976. In detail, high yields of L-threonine can be achieved by employing a recombinant microorganism, which possesses on chromosome

one or more copies of a gene encoding phosphoenol pyruvate carboxylase (hereinafter, referred to simply as "*ppc*") that catalyzes the formation of oxaloacetate (OAA) as the precursor for L-threonine biosynthesis from phosphoenol pyruvate (PEP) and an operon including genes encoding the three enzymes participating in L-threonine biosynthesis from aspartate, aspartokinase 1-homoserine dehydrogenase (*thrA*), homoserine kinase (*thrB*) and threonine synthase (*thrC*).

L-threonine is synthesized from asparate by a multi-step pathway, wherein the aspartate is formed from OAA converted by PPC from PEP. L-threonine biosynthesis is inhibited when glucose is present in relatively high levels in media in comparison with the bacterial growth rate and the overall rate of the TCA cycle. In this situation, the *ppc* gene expression is suppressed, while expression of a gene encoding PEP carboxykinase (hereinafter, referred to simply as "*pckA*") catalyzing the conversion of OAA into PEP is increased. The elevated levels of *pckA* result in the formation of PEP from OAA as the precursor for amino acid biosynthesis, wherein other by-products are synthesized from the PEP (Goldie H. Medina V., *Mol. Gen. Genet.*, 220(2):191-196, 1990; Dang et al., *E.coli and Salmonella*, 1:191-102, 1996). Therefore, the *pckA* gene should be essentially inactivated in order to produce L-threonine in high levels by increasing the flux of metabolic pathways responsible for L-threonine synthesis.

On the other hand, several pathways for L-threonine degradation are known, which include the following three pathways. One involves a pathway initiated by threonine dehydrogenase yielding α-amino-β-ketobutyrate. The α-amino-β-ketobutyrate is either converted to acetyl-CoA and glycine or spontaneously degrades to aminoacetone that is converted to pyruvate. The second pathway involves threonine dehydratase yielding α-ketobutyrate which is further catabolized to propionyl-CoA and finally the TCA cycle intermediate, succinyl-CoA. The third pathway utilizes threonine aldolase (Neidhardt F.C. et al. *Escherichia coli and Salmonella: cellular and molecular biology*, 2nd ed. ASM press. Washington DC, pp369-370). Among them, the threonine dehydratase is an operon that is expressed under hypoxia and high levels of threonine.

The present inventors developed a microorganism with improved productivity of L-threonine by specifically inactivating this operon gene (*tdcBC*) via a genetic recombination technique (Korean Pat. Application No. 2002-015380).

On the other hand, International Pat. Publication No. WO 02/29080 A2 discloses a method of producing L-threonine using a *pckA* gene-defective microorganism, which is prepared by introducing it into a wild type strain of the microorganism a recombinant vector carrying a partially deleted *pckA* gene. However, this microorganism is problematic with respect to production yield of L-threonine because pathways for degradation and intracellular influx of synthesized L-threonine are still activted in the microorganism.

Leading to the present invention, the intensive and thorough research conducted by the present inventors into methods of preparing a microorganism capable of producing high levels of L-threonine even when grown in a medium containing high concentrations of glucose and not degrading L-threonine produced, with aim to solve the problems encountered in the prior art, resulted in the finding that, when its chromosomal *pckA* gene is inactivated by a genetic recombination technique, the *tdcBC* operon - knocked out microorganism developed by the present inventors has improved L-threonine productivity in comparison with the conventional L-threonine-producing microorganisms.

It is therefore an object of the present invention to provide a microorganism capable of effectively producing high levels of L-threonine.

SUMMARY OF INVENTION

In order to accomplish the above object, the present invention provides a novel strain of *E. coli*, which contains both *tdcBC* and *pckA* genes inactivated on chromosome.

In the *tdcBC*/*pckA* gene-inactivated *E. coli* strain, the *pckA* gene is inactivated

by introducing a foreign *pckA* gene fragment containing an antibiotic resistance gene and a site-specific recombinase binding site at each of its both ends into an *E. coli* strain containing an L-threonine degradation-associated operon, *tdcBC* that is inactivated, and then allowing for homologous recombination between the foreign *pckA* gene fragment and a *pckA* gene on chromosome to inactivate the chromosomal *pckA* gene.

In addition, the present invention provides a method of producing L-threonine using the *tdcBC/pckA* gene-inactivated *E. coli* strain.

BRIEF DESCRIPTION OF THE DRAWINGS

The above and other objects, features and other advantages of the present invention will be more clearly understood from the following detailed description taken in conjunction with the accompanying drawings, in which:

FIG. 1 is a schematic view showing a process of cloning a *pckA* gene;

FIG. 2 is a schematic view showing a process of preparing a recombinant microorganism into which a *pckA* gene fragment containing a chloramphenicol resistance gene *(cat)* and loxP sites, ΔpckA::loxpcat, is introduced; and

FIG. 3 is a photograph showing a result of Southern blotting, in which a chloramphenicol resistance gene*(cat)* is identified to be inserted into a *pckA* gene on the chromosome of an L-threonine-producing *E. coli* strain (lane 1: recombinant strain selected in the presence of chloramphenicol according to the present invention; lane 2: parent strain TRN212; and lane 3: size marker).

DETAILED DESCRIPTION OF THE INVENTION

A strain of *E. coli*, which contains an L-threonine degradation-associated operon specifically inactivated by genetic recombination and has improved productivity of L-threonine due to the inactivation of the operon, may be used as a parent strain in

the present invention. A preferred parent strain is an *E. coli* strain TRN212 (accession number: KCCM-10353; Korean Pat. Application No. 2002-015380), which was developed by the present inventors.

The present invention is characterized by preparing a novel *E. coli* strain producing high levels and high yields of L-threonine by inactivating the *pckA* gene involved in inhibition of L-threonine synthesis in a parent *E. coli* strain containing an L-threonine degradation-associated operon (*tdcBC*) inactivated. The inactivation of both *tdcBC* and *pckA* genes results in the prevention of degradation and intracellular influx of L-threonine, mediated by translational products of the *tdcBC* operon, and the inhibition of L-threonine synthesis, mediated by a translational product of the *pckA* gene, leading to high level production of L-threonine.

Therefore, the present invention provides a *tdcBC/pckA* gene-inactivated *E. coli* strain, which is prepared by introducing a foreign *pckA* gene fragment including an antibiotic resistance gene having a site-specific recombinase binding site at each of its both ends into an *E. coli* strain containing an L-threonine degradation-associated operon, *tdcBC*, that is inactivated, and then allowing for homologous recombination between the foreign *pckA* gene fragment and a *pckA* gene on chromosome to inactivate the chromosomal *pckA* gene.

In addition, the *pckA* gene on chromosome of the parent *E. coli* strain is inactivated by removal of the antibiotic resistance gene incorporated into the chromosomal *pckA* gene by the activity of the site-specific recombinase expressed in the bacterial strain, and the presence of one copy of a binding site of the site-specific recombinase in the chromosomal *pckA* gene.

The inactivation of the *pckA* gene on the bacterial chromosome is achieved by homologous recombination with a foreign *pckA* gene fragment. The foreign *pckA* gene fragment is inactivated by insertion of an antibiotic resistance gene thereinto. This foreign inactivated *pckA* gene fragment is introduced into a parent *E. coli* strain, and double crossover recombination is then allowed to occur between a *pckA* gene on

the bacterial chromosome and the foreign inactivated *pckA* gene fragment to inactivate the *pckA* gene on the bacterial chromosome. The presence of the antibiotic resistance gene in the foreign inactivated *pckA* gene facilitates selection of *pckA* gene-inactivated cells.

Non-limiting examples of the antibiotic resistance gene used in the inactivation of the *pckA* gene include chloramphenicol resistance gene, kanamycin resistance gene, gentamycin resistance gene and ampicillin resistance gene.

On the other hand, after a *pckA* gene-inactivated *E. coli* strain is selected, a site-specific recombinase is allowed to be expressed to remove the antibiotic resistance gene incorporated into the bacterial chromosome. That is, the antibiotic resistance gene is incorporated into the *pckA* gene on the bacterial chromosome along with site-specific recombinase binding sites, and removed by the activity of the site-specific recombinase expressed in the bacterial strain. Non-limiting examples of the site-specific recombinase include binding sites of FLP, Cre and XerC/D. The removal of the antibiotic resistance gene allows the same antibiotic resistance gene to be used again as a selective marker when another gene of the identical bacterial strain is desired to be inactivated.

In the present invention, in order to inactivate the chromosomal *pckA* gene, a *pckA* gene fragment containing a chloramphenicol resistance gene each end of which is linked to a loxP site is used. The loxP site is recognized by a site-specific recombinase, Cre. By the activity of Cre recombinase expressed in the *E. coli* strain, the antibiotic resistance gene located between the two loxP sites is removed from the bacterial chromosome.

The Cre recombinase expression in the *E. coli* strain may be achieved by a method known in the art. In the present invention, a plasmid carrying a *cre* gene, pJW168, is introduced into the *E. coli* strain to express Cre enzyme therein.

In one embodiment of the present invention, a partial *pckA* gene was amplified by PCR using as a template genomic DNA isolated from a L-threonine-producing *E.*

coli strain including a *tdcBC* operon inactivated. The amplified partial *pckA* gene was cloned into a pT7Blue vector (Novagen Co.), thus yielding a recombinant vector containing a partial *pckA* gene, pT7Blue/pckA. In addition, a DNA fragment containing a chloramphenicol resistance gene and loxP sites, loxpcat2, was obtained from a ploxpcat2 plasmid (Beatriz Palmeros et al., *Gene*, 247:255-264, 2000), and ligated to NruI-digested pT7Blue/pckA, thus generating a recombinant plasmid containing a *pckA* gene fragment including a chloramphenicol resistance gene and loxP sites, pT7ΔpckA::loxpcat. Therefore, the present invention provides the recombinant plasmid as prepared above, pT7ΔpckA::loxpcat.

In another embodiment of the present invention, the *pckA* gene fragment containing a chloramphenicol resistance gene each end of which is linked to a loxP site was introduced into an *E. coli* strain TRN212 containing a *tdcBC* operon that is inactivated by homologous recombination using a kanamycin resistance gene having a loxP site at each of its both ends. Then, homologous recombination was allowed to occur between a *pckA* gene on the bacterial chromosome and a foreign *pckA* gene fragment containing the chloramphenicol resistance gene and the loxP sites, thus yielding a recombinant *E. coli* strain containing both a *tcdBC* gene and a *pckA* gene inactivated on chromosome. The recombinant *E. coli* strain was designated as "FTR2717", and deposited at the Korean Culture Center of Microorganisms (KCCM) on March 20, 2003 under an accession number of KCCM-10475.

The recombinant *E. coli* FTR2717 strain exhibits the following characteristics:

(1) it has resistance to threonine analogues, lysine analogues, isoleucine analogues and methionine analogues in comparison with a wild type thereof;

(2) its chromosome contains an endogenous *ppc* gene and an endogenous threonine operon containing *thrA*, *thrB* and *thrC* genes as well as one or more copies of an exogenous *ppc* gene and exogenous *thrA*, *thrB* and *thrC* genes;

(3) it includes an operon gene involved in L-threonine degradation, *tdcBC*, which is inactivated; and

(4) it includes a *pckA* gene involved in inhibition of L-threonine synthesis, which is inactivated, so that it produces high levels of L-threonine under a high concentration of glucose in a medium.

A better understanding of the present invention may be obtained through the following examples which are set forth to illustrate, but are not to be construed as the limit of the present invention.

EXAMPLE 1: Cloning of *pckA* gene

A recombinant vector carrying a *pckA* gene was prepared (see, FIG. 1). First, bacetrial genomic DNA was isolated from a L-threonine-producing *E. coli* strain including a *tdcBC* operon inactivated, TRN212 (accession number: KCCM-10353), using a QIAGEN Genomic-tip system (QIAGEN Co.). Using the isolated genomic DNA as a template, PCR was carried out to amplify an about 1.5-kb partial region of a *pckA* gene. In the PCR, a primer set was used, which consists of a forward primer and a reverse primer represented by SEQ ID NOs: 1 and 2, respectively. PCR conditions included 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 1 min 30 sec.

The PCR products were electrophoresed on a 0.8% agarose gel, and a 1.5-kb band was excised out from the gel. From the excised band, a 1.5-kb DNA fragment was purified using a DNA Gel Purification Kit (QIAGEN Co.), and cloned into an EcoRV-digested pT7Blue vector (Novagen Co.) by blunt end ligation at 16°C, thus yielding a recombinant vector containing a partial *pckA* gene, pT7Blue/pckA. Then, an *E. coli* NM522 strain was transformed with the pT7Blue/pckA, and smeared on a solid medium (LB: 1% NaCl, 1% Tryptone, 0.5% Yeast extract) containing ampicillin (100 mg/L), followed by incubation at 37°C overnight. The colonies grown on the solid medium were inoculated in 3 ml of a liquid medium containing ampicillin,

followed by incubation at 37°C overnight. Plasmid DNA was isolated from the cultured bacteria using a QIAGEN mini prep kit (QIAGEN Co.), and analyzed for its size. Also, orientation of the *pckA* gene was analyzed by restriction mapping with NruI and StuI. Thereafter, the plasmid DNA was digested with NruI, and electrophoresed on a 0.7% agarose gel. From the gel, an about 4.3-kb band was excised, and a 4.3-kb fragment was purified from the band.

EXAMPLE 2: Construction of recombinant vector carrying an inactivated *pckA* gene and preparation of *pckA* gene-inactivated *E. coli* strain

2-1) Construction of a recombinant vector carrying an inactivated *pckA* gene

A 1.2-kb DNA fragment, loxpcat, which contains a chloramphenicol resistance gene having a loxP site at each of its both ends was obtained by digesting with HincII a ploxpcat2 plasmid (plasmid carrying a chloramphenicol resistance gene having loxP sites at its both ends; Beatriz Palmeros et al., *Gene*, 247:255-264, 2000, Professor G. Gosset, University of Mexico). The 1.2-kb DNA fragment was ligated to the NruI-digested pT7Blue/pckA prepared in Example 1 by blunt end ligation, thus yielding an about 5.7-kb recombinant vector containing an inactivated *pckA* gene, pT7ΔpckA::loxpcat (see, FIG. 2).

2-2) Preparation of a *pckA* gene-inactivated *E. coli* strain

The pT7ΔpckA::loxpcat recombinant vector, prepared in Example 2-1), was introduced into an *E. coli* NM522 strain. The transformed NM522 strain was smeared on a solid medium (LB: 1% NaCl, 1% Tryptone, 0.5% Yeast extract) containing ampicillin and chloramphenicol, followed by incubation at 37°C overnight. The colonies grown on the solid medium were inoculated in 3 ml of a liquid medium containing ampicillin and chloramphenicol, followed by incubation at 37°C overnight. Plasmid DNA was isolated from the cultured bacteria using a QIAGEN mini prep kit,

and analyzed for its size and orientation of the inserted *pckA* gene. Thereafter, the plasmid DNA was double-digested with PstI and KpnI, and electrophoresed on a 0.7% agarose gel. From the gel, a 2.7-kb band was excised, and a 2.7-kb fragment (ΔpckA::loxpcat) was purified from the band.

The *pckA* gene fragment containing a chloramphenicol resistance gene having loxP sites at its both ends, ΔpckA::loxpcat, was introduced into a L-threonine-producing *E. coli* strain, TRN212 (accession number: KCCM-10353), by electroporation. Thereafter, the transformed TRN212 strain was smeared on a solid medium containing chloramphenicol to select only chloroamphenicol-resistant cells, resulting in selection of cells wherein a *pckA* gene on chromosome was replaced with the foreign *pckA* gene fragment (ΔpckA::loxpcat). The selected clones were evaluated for whether the chromosomal *pckA* gene is specifically knocked out, by Southern blot analysis according to the same method as in Experimental Example 1, below.

The selected clones, which were identified to have a *pckA* gene specifically knocked out on chromosome by Southern blot analysis, were transformed with a pJW168 plasmid (gift from Prof. Guillermo Gosset at the University of Mexico) that contains a *cre* gene encoding a site-specific recombinase recognizing loxP sites. The transformed cells were cultured in a culture medium containing 10 mM L-arabinose overnight to remove the chloramphenicol resistance gene incorporated int o the bacterial chromosome. Then, the culture fluid was diluted 10^7-fold and smeared on a LB solid medium supplemented with ampicillin (100 mg/L), followed by incubation at 30°C overnight. Each of 100 colonies grown on the solid medium was inoculated in 3 ml of each of LB liquid media containing ampicillin or not, followed by incubation at 30°C overnight. Colonies that were killed in the medium containing chloramphenicol but survived in the medium not containing chloramphenicol were determined. In this selection, only clones having a deletion of the chloramphenicol resistance gene were selected.

EXPERIMENTAL EXAMPLE 1: Evaluation of knock-out of *pckA* gene on chromosome by Southern blotting

The TRN212 strain as a parent strain and one of the chloroamphenicol-resistant clones selected in Example 2-2) were cultured overnight in 3 ml of a liquid medium containing chloramphenicol (15 mg/L). Then, genomic DNA was isolated from the culture cells using a QIAGEN genomic kit 20, and was digested with EcoRV overnight. The resulting DNA fragments were separated on a 0.7% agarose gel depending on their size. After electrophoresis, the separated DNA fragments were transferred onto a nylon membrane (Biodyne B membrane, Young Sci.) overnight by capillary transfer (Molecular Cloning, Vol 1., pp6.31-6.38). The membrane was dried and then exposed to an UV light (120 mJ/cm^2, SpectroLinker^{TM}) to immobilize the DNA fragments on the membrane (Molecular Cloning, Vol 1., pp6.45). The resulting membrane was incubated in a prehybridization solution I (Roche #1093657) at 55°C for 2 hrs, and hybridized with a denatured DNA probe overnight in a hybridization oven (BAMBINO 230300) at 55°C.

The DNA probe was prepared as follows. First, a ploxpcat2 plasmid was isolated using a QIAGEN kit and digested with HincII to yield a DNA fragment (about 1.2 kb) containing a chloramphenicol resistance gene having a loxP site at each of its both ends. The 1.2-kb fragment was boiled in water for 5 min and quick-cooled on ice, thus yielding a single-stranded DNA. The single-stranded DNA was then labeled with DIG-UDP using a DIG Labeling and Detection Kit (Roche #1093657) by incubation at 37°C overnight.

After hybridization, the membrane was washed with washing solutions I and II (Roche #1093657) to remove non-specifically attached DNA molecules. The washed membrane was masked using a prehybridization solution II (Roche #1093657) at room temperature for 30 min, and then reacted with an anti-DIG antibody specifically binding to DIG-UTP at room temperature for 30 min. The membrane was washed with a

washing solution III (Roche #1093657) to remove non-specifically attached anti-DIG antibodies, and developed using a Labeling and Detection Kit (Roche #1093657) at room temperature until bands were emerged. The results are given in FIG. 3.

As shown in FIG. 3, in case of the parent strain TRN212, no band was detected (lane 2) because the TRN212 strain did not contain a chloramphenicol resistance gene. In contrast, the chloramphenicol-resistant clone selected according to the present invention showed an about 3.6-kb band (lane 1). These results indicate that the selected clones contain a chloramphenicol resistance gene on its chromosome.

EXAMPLE 3: Comparison of the selected clones for production yields of L-threonine upon culturing in Erlenmeyer flasks

Among the finally selected recombinant *E. coli* clones of Example 2-2) in which the introduced chloramphenicol resistance gene was removed, thirty clones were evaluated for productivity of L-threonine. Each of them was cultured in an Erlenmeyer flask containing a culture medium prepared according to the composition listed in Table 1, below. Then, each culture fluid was evaluated for production yields of L-threonine. In brief, after each of the thirty clones were grown on a LB solid medium at 32°C, one loop of a single colony for each clone was inoculated in 25 ml of the culture medium and cultured at 32°C for 48 hrs at 250 rpm. After each of the culture fluids was centrifuged, the supernatant was 250-fold diluted with distilled water. L-threonine concentration in the diluted supernatant was measured by HPLC. The results are given in Table 2, below.

TABLE 1

Nutrients	Amount per 1 L
Glucose	70 g

Ammonium sulfate	28 g
KH_2PO_4	1.0 g
$MgSO_4 \cdot 7H_2O$	0.5 g
$FeSO_4 \cdot 7H_2O$	5 mg
$MnSO_4 \cdot 8H_2O$	5 mg
Calcium carbonate	30 g
L-methionine	0.15 g
Yeast extract	2 g
pH (7.0)	

TABLE 2

The number of clones	2	5	14	9
Production yield of L-threonine (g/L)	20-23	23-24.5	24.5-26	>26

The parent strain TRN212 showed a L-threonine production yield of 23 g/L. Among the thirty tested clones, twenty-eight were found to have better productivity of L-threonine than the TRN212 strain, as shown in Table 2. In particular, nine clones showed a L-threonine production yield higher than 26 g/L, which was about 13.04% higher than the yield of the TRN212 strain. Among the thirty clones, one clone with the highest yield of L-threonine (over 26 g/L) was selected and designated as "FTR2717 (accession number: KCCM-10475)".

INDUSTRAL APPLICABILITY

As described hereinbefore, the present invention provides a *pckA* gene-inactivated microorganism, which is prepared by introducing an antibiotic resistance gene into the chromosomal DNA of a parent *E. coli* strain producing high levels of L-threonine, that is, an *E. coli* strain containing a *tdcBC* operon involved in the degradation of L-threonine, which is inactivated, by a DNA recombination technique.

Since its chromosomal *tdcBC* operon is inactivated, the microorganism according to the present invention has the effect of preventing degradation and intracellular influx of L-threonine. In addition, due to the inactivation of the *pckA* gene involved in the inhibition of L-threonine synthesis, the microorganism of the present invention has more activated pathways for L-threonine biosynthesis. Therefore, the microorganism of the present invention is useful for mass production of L-threonine because of being capable of producing L-threonine in high levels and high yields even in the presence of high concentrations of glucose.

Applicant's or agent's File reference PCTA9403-3	International application No.

INDICATIONS RELATING TO DEPOSITED MICROORGANISM

OR OTHER BIOLOGICAL MATERIAL

A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page 08, line 19-20

B. IDENTIFICATION OF DEPOSIT Further deposits are on an additional sheet[...]

Name of depositary institution

Korean Culture Center of Microorganisms

Address of depositary institution (*including postal code and country*)

361-221, Yurim B/D, Hongje-1-dong, Seodaemun-gu, Seoul 120-091, Republic of Korea

Date of deposit	Accession Number
20/03/2003	KCCM-10475

C. ADDITIONAL INDICATIONS (*leave blank if not applicable*) This information is continued on an additional sheet [ ]

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (*if the indications are not for all designated States*)

E. SEPARATE FURNISHING OF INDICATIONS (*leave blank if not applicable*)

The indications listed below will be submitted to the International Bureau later (*specify the general nature of the indications e.g., "Accession Number of Deposit"*)

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Authorized officer	Authorized officer

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What is claimed is:

1. An *Escherichia coli* strain comprising both *tdcBC* and *pckA* genes that are inactivated.

2. The *Escherichia coli* strain as set forth in claim 1, wherein the *pckA* gene is inactivated by introducing a foreign *pckA* gene fragment containing an antibiotic resistance gene having a site-specific recombinase binding site at each of both ends thereof into a parent *Escherichia coli* strain containing an L-threonine degradation-associated operon, *tdcBC*, that is inactivated, and then allowing homologous recombination between the foreign *pckA* gene fragment and the *pckA* gene on chromosome to inactivate the chromosomal *pckA* gene.

3. The *Escherichia coli* strain as set forth in claim 2, wherein the *pckA* gene is inactivated by removal of the antibiotic resistance gene incorporated thereinto by the activity of the site-specific recombinase expressed in the *Escherichia coli* strain and the presence of one copy of the binding site of the site-specific recombinase in the chromosomal *pckA* gene.

4. The *Escherichia coli* strain as set forth in claim 2, wherein the site-specific recombinase is FLP, Cre or XerC/D.

5. The *Escherichia coli* strain as set forth in claim 2, wherein the strain is *Escherichia coli* FTR2717 (KCCM-10475) comprising on chromosome a *pckA* gene inactivated by introducing a foreign *pckA* gene fragment containing an antibiotic resistance gene having a loxP site at each of both ends thereof into the parent *Escherichia coli* strain containing the L-threonine degradation-associated operon, *tdcBC*, that is inactivated.

6. A method of producing L-threonine using the *Escherichia coli* strain of any one of claims 1 to 5.

7. A recombinant plasmid pT7ΔpckA::loxpcat comprising a *pckA* gene fragment including a chloramphenicol resistance gene and loxP sites, wherein the recombinant plasmid is prepared by cloning a partial *pckA* gene into a pT7Blue vector to produce a pT7Blue/pckA plasmid, obtaining a DNA fragment containing a chloramphenicol resistance gene and loxP sites, loxpcat2, from a ploxpcat2 plasmid, and ligating the loxpcat DNA fragment to the NruI-digested pT7Blue/pckA plasmid.

ABSTRACT

Disclosed is a microorganism including both *tdcBC* and *pckA* genes inactivated on chromosome, which has remarkably improved productivity of L-thionine due to the inactivation of the two genes. Also, the present invention discloses a method of producing L-threonine using the microorganism. The microorganism is prepared by incorporating by a recombination technique an antibiotic resistance gene into a *pckA* gene on the chromosome of a bacterial strain containing an L-threonine degradation-associated operon gene, *tdcBC*, which is inactivated. The microorganism has the effect of preventing degradation and intracellular influx of L-threonine due to the inactivation of the *tdcBC* operon gene, and includes more activated pathways for L-threonine biosynthesis. Therefore, the microorganism is useful for mass production of L-threonine because of being capable of producing L-threonine in high levels and high yields even in the presence of high concentrations of glucose.